Obesity Up-Regulates Intermediate Conducance Calcium-Activated Potassium Channels and Myoendothelial Gap Junctions to Maintain Endothelial Vasodilator Function

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Received February 24, 2010; accepted July 28, 2010

ABSTRACT

The mechanisms involved in altered endothelial function in obesity-related cardiovascular disease are poorly understood. This study investigates the effect of chronic obesity on endothelium-dependent vasodilation and the relative contribution of nitric oxide (NO), calcium-activated potassium channels (K_Ca), and myoendothelial gap junctions (MEGJs) in the rat saphenous artery. Obesity was induced by feeding rats a cafeteria-style diet (~30 kJ as fat) for 16 to 20 weeks, with this model reflecting human dietary obesity etiology. Age- and sex-matched controls received standard chow (~12 kJ as fat). Endothelium-dependent vasodilation was characterized in saphenous arteries by using pressure myography with pharmacological intervention, Western blotting, immunohistochemistry, and ultrastructural techniques. In saphenous artery from control, acetylcholine (ACh)-mediated endothelium-dependent vasodilation was blocked by NO synthase and soluble guanylate cyclase inhibition, whereas in obese rats, the ACh response was less sensitive to such inhibition. Conversely, the intermediate conductance K_Ca (I_KCa) blocker 1-[{(2-chlorophenyl)diphenyl-methyl]-1H pyrazole attenuates ACh-mediated dilation in obese, but not control, rats. IK1 protein and MEGJ expression was up-regulated in the arteries of obese rats, an observation absent in control. Addition of the small conductance K_Ca blocker apamin had no effect on ACh-mediated dilation in either control or obese rat vessels, consistent with unaltered SK3 expression. Up-regulation of distinct I_KCa and gap junction-mediated pathways at myoendothelial microdomain sites, key mechanisms for endothelial-derived hyperpolarization-type activity, maintains endothelium-dependent vasodilation in diet-induced obese rat saphenous artery. Plasticity of myoendothelial coupling mechanisms represents a significant potential target for therapeutic intervention.

Introduction

Diet-induced obesity is at epidemic levels, with hypertension, hyperglycemia, and insulin resistance being a common outcome (Haslam and James, 2005). The vascular endothelium modulates vasodilator and vasoconstrictor action and is therefore critical for the control of vascular tone and disease etiology. Endothelium-dependent vasodilation depends on nitric oxide (NO), prostacyclin (PGL2), and the non-NO/PGL2 endothelial-derived hyperpolarization (EDH) mechanism (reviewed in McGuire et al., 2001; Sandow and Tare, 2007; Sandow et al., 2009b).

Key events in the EDH process involve an agonist-induced increase in endothelial cell intracellular calcium and activa-
tion of small conductance calcium-activated potassium channels (SKCa; SK3; KCa2.3(SKCa3/KCNN3) and intermediate conductance calcium-activated potassium channels (IKCa; IK1; SK4/KCa3.1(KCNN4). This leads to the subsequent hyperpolarization of the smooth muscle via myoendothelial gap junction (MEGJ)-mediated transfer of current and/or K⁺ release and activation of inward rectifying potassium channels and/or smooth muscle Na⁺/K⁺ ATPase (Hill, 2008; Félotou, 2009; Sandow et al., 2009b). The relationship between MEGJs and EDH activity is supported by studies where gap junction “block” with connexin (Cx)-mimetic “Gap” peptides (Chaytor et al., 2001; Sandow et al., 2004; 43Gap26, 40Gap27, and 37,48Gap27) or licorice derivatives such as carbenoxolone attenuate EDH-mediated vasodilation (Coleman et al., 2001). In rat mesenteric artery, myoendothelial microdomain IKCa and MEGJs show close spatial association (Sandow et al., 2006, 2009b) that is functionally associated with their activity in EDH (Dora et al., 2008; Félotou, 2009; Sandow et al., 2009b).

Altered endothelial function, like heterogeneity in mechanisms of vascular function in general (Aird, 2007), involves a differential contribution of vasoconstrictor and vasodilator activity to the control of vessel tone (Aird, 2007). The specific action of these stimuli varies within and between beds, disease, development, aging, species, strains, sex, and, unfortunately, experimental methodology (Sandow et al., 2009b). In a similar manner, altered endothelium-dependent vasodilation involves changes in the relative contribution of NO, PGI2, and EDH. Indeed, the contribution of IKCa subtypes and MEGJs to EDH also shows an apparent variation in this manner (McGuire et al., 2001; Sandow et al., 2009b), with alterations in specific such components in pathophysiological states, such as obesity, perhaps contributing to the maintenance of vascular reactivity.

In some forms of vascular disease, altered vessel constriction and/or dilation is associated with reduced NO bioavailability, with EDH acting as a backup pathway to sustain vessel diameter (Taddei et al., 2001). Altered endothelial function is associated with animal models of type I diabetes (Shi et al., 2006), type II diabetes (Park et al., 2008), pulmonary hypertension (Kemp et al., 1995), hypercholesterolemia (Brandes et al., 1997), heart failure (Katz and Krum, 2001), and aging (Gaubert et al., 2007), where NO activity is reduced, and KCa-mediated EDH is preserved or up-regulated to maintain overall vasodilation. The disease-related effects of endothelium-dependent vasodilation in diet-induced obesity are unknown.

In adult rat saphenous artery, endothelium-dependent vasodilation is entirely NO-mediated (Wigg et al., 2001; Sandow et al., 2002, 2004). The inability of an EDH to be transferred to the adjacent smooth muscle is caused by the lack of sufficient MEGJs in this bed to facilitate current/ion transfer between the cell layers (Sandow et al., 2002, 2004), which contrasts with the saphenous artery of juvenile rat, where MEGJ-dependent EDH is down-regulated after development (Sandow et al., 2004).

Using a well characterized rodent model of diet-induced obesity associated with elevated blood pressure, insulin, and leptin that directly reflects the etiology human dietary obesity (Velkoska et al., 2005; Chen et al., 2009), the effect of chronic obesity on endothelium-dependent vasodilation in the small saphenous artery was examined. The hypothesis tested was that the mechanism of endothelium-dependent vasodilation would be altered in obesity-induced vascular disease, with KCa and MEGJ components being up-regulated.

### Materials and Methods

#### Animals

Animal procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the University of New South Wales Animal Care and Ethics Committee.

Eight-week-old male Sprague-Dawley rats were fed normal chow (control); 12% kJ as fat) or a cafeteria-style high-fat diet (obese; chow supplemented with condensed milk and lard, biscuits, cakes, dim sims, meat pies, and pasta; 30% kJ as fat) for 16 to 20 weeks. High-fat and control diets were available ad libitum throughout, with nonfasted glucose, leptin, and insulin being measured immediately upon sacrifice. At 24 to 28 weeks (~6 months) of age, obese rats were significantly heavier than the chow-fed control age-matched animals and were characterized by increased liver, retroperitoneal fat, and testicular fat mass. In addition, obese rats had significantly higher levels of blood glucose, leptin, and insulin (Table 1).

#### Artery Preparation

Rats were anesthetized with sodium pentathol (100 mg · kg⁻¹ i.p.), and vascular beds (saphenous and femoral) from the lower hind limb were removed and placed in cold Krebs' solution containing 112 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgSO4, 7 mM H2PO4, 0.7 mM KH2PO4, 10 mM HEPES, 11.6 mM glucose, 2.5 mM CaCl2, 2 mM H2O2, pH 7.3. The first lateral branch of the saphenous artery that diverged toward the knee (control, 330 ± 7 μm, n = 25; obese, 349 ± 5 μm, n = 43) was isolated and carefully cleaned of connective tissue.

#### Pressure Myography

Saphenous arteries were cannulated in a pressure myograph and continuously superfused with Krebs' solution (37°C) at a rate of 2 ml/min. Arteries were pressurized to 80 mm Hg with incremental increases over 80 min. Vessels were initially preconstricted with superfused phenylephrine (1 μM; to 80% of maximum constriction) before increasing concentrations of acetylcholine (ACH; 1 μM to 30 μM) were added to the bath. Experiments were conducted in the presence and absence of selective blockers of endothelium-dependent vasodilator pathways (see Drugs). In some experiments, SKCa and IKCa blockers, apamin (1-[(2-chlorophenyl)diphenyl-methyl]-1H pyrazole (TRAM-34), respectively, and SKCa and IKCa agonists, cyclohexyl[2,4,5,6]-dimethyl-pyrazol-1-yl]-6-methyl-pyrimidin-4-yl]-amine (CyPPA; 0.01–30 μM) and 1-ethyl-2-benzimidazolodizalone (1-EBIO; 1–300 μM), respectively, were added to the bath after preconstriction, the latter in the presence and absence of TRAM-34, with TRAM-34 therefore effectively acting as a control for 1-EBIO specificity. Because of the absence of an apamin-sensitive vasodilatory component in these vessels, similar CyPPA experiments were not conducted. Artery diameter changes are expressed as a

#### TABLE 1

Control and diet-induced obese rat, organ, and metabolic characteristics (5-week-old male rats fed control/high-fat diet for 16–20 weeks; total age, 24–28 weeks)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Weight (g)</td>
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</tr>
<tr>
<td></td>
<td>Length (cm)</td>
<td>25.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Retropertioneal (g)</td>
<td>6.4 ± 0.3</td>
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<td>Kidney (g)</td>
<td>1.6 ± 0.03</td>
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<tr>
<td></td>
<td>Liver (g)</td>
<td>17.8 ± 0.5</td>
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<tr>
<td></td>
<td>Testicular (g)</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Blood glucose (mM)</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Leptin (ng/ml)</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Insulin (ng/ml)</td>
<td>4.6 ± 0.7</td>
</tr>
</tbody>
</table>

*P < 0.05, indicates significant difference from control.
percentage of the maximal dilatation achieved by replacing the control Krebs’ solution with [Ca\(^{2+}\)]\_o-free Krebs’ solution.

**Western Blotting.** Arteries were dissected from both hind legs of age-matched control and obese rats, extraneous tissue was carefully removed, and the arteries were stored in liquid nitrogen. Vessels from four animals per n (i.e., per sample lane; n = 3 and 4, for obese and control groups, respectively) were collected. These arteries were ground in liquid nitrogen with a pestle and mortar, resuspended in phosphate-buffered saline (PBS), pH 7.4 containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and centrifuged (3000g; 4°C, 5 min). The supernatant was removed and placed on ice, and the pellet was snap-frozen in liquid nitrogen and processed again as described above. After the second spin the supernatants were pooled and centrifuged (25,000g; 4°C; 1 h), and the supernatant, enriched in cytosolic proteins, was aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. The membrane-enriched pellet was carefully resuspended in PBS containing 0.1% Triton X-100 and protease inhibitor cocktail, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. Protein concentration of the samples was determined by using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Aliquots of protein extracts (5 μg of protein unless otherwise indicated) were dissolved in lithium dodecyl sulfate sample buffer (0.5% lithium dodecyl sulfate, 62.5 mM Tris-HCl, 2.5% glycerol, 0.125 mM EDTA, pH 8.5) for 10 min at 70°C. The samples were separated by electrophoresis in bis-Tris polyacrylamide gels by using MES SDS running buffer and electroblotted onto polyvinylidene difluoride membranes overnight at 4°C, according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). After transfer, blots were thoroughly washed, blocked, and probed with primary antibody, and specific binding was visualized by using alkaline phosphatase-conjugated secondary antibody and chemiluminescence according to the manufacturer’s instructions (Invitrogen). The intensity of the band corresponding to each protein was quantified by digital densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Relative intensity for each protein was determined by comparison with the intensity of actin staining on blots that were stripped and then reprobed with actin primary antibody (Supplemental Fig. S1).

To determine specificity, each antibody was incubated with its cognate peptide to block specific binding. Before use, peptide was added to antibody in a 1:1 ratio (w/w), mixed, and incubated at 37°C for 1 h, then overnight at 4°C. The blocked antibody was then used in Western blotting detection as described above.

**Immunohistochemistry.** The distribution of SK\(_{Ca}\) and IK\(_{Ca}\) was examined in the rat saphenous artery by using conventional whole-mount confocal immunohistochemistry (Mather et al., 2005; Hadcock et al., 2006). Rats were perfused via the left ventricle with a clearing solution of 0.1% bovine serum albumin, 0.1% NaNO\(_3\) and 10 U/ml heparin to fully dilate the vessels and fixed with 2% paraformaldehyde in 0.1 M PBS. Tubular vessel segments were cut along the lateral plane and pinned out as a flat sheet with the intima uppermost.

Whole-mount tissues were incubated in blocking buffer (PBS containing 1% bovine serum albumin, 0.2% Tween 20) for 2 h at room temperature, rinsed in PBS (3 × 5 min), and incubated in primary antibody (SK\(_{Ca}\) as SK3, 1:100, Mark Chen, GlaxoSmithKline, Stevenage, United Kingdom, M75; IK\(_{Ca}\), as IK1, 1:100, Mark Chen, M20; Supplemental Table S2; compared with those exhibiting less specificity, Supplemental Table S3) in blocking buffer for 2 h. It was then rinsed in PBS (3 × 5 min) and incubated in secondary antibody (Alexa Fluor 633; Invitrogen) diluted in 0.01% Tween 20 for 2 h. It was then rinsed in PBS (3 × 5 min), mounted intima uppermost in antifade glycerol, and examined with a confocal microscope (PV1000; Olympus, Tokyo, Japan) using uniform settings. CellIR software (Olympus) was used for quantitative measurements, with n being the number of observations, each from a different animal, from four different randomly selected 10\(^{-3}\)mm\(^2\) areas per animal (see Figs. 3C and 6E). Controls for antibody specificity involved abolition of staining after preincubation of SK3 (M75) and IK1 (M20) antibodies with their respective antigenic peptides (see Fig. 2B; Supplemental Fig. S2), omission of the primary antibody, and the use of transfected cells, immunoelectron microscopy, and positive controls for SK3 (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005; Sandow et al., 2006).

**Electron Microscopy.** Tissue preparation and analysis for serial section electron microscopy were as described previously (Sandow et al., 2002, 2004). In brief, control and obese saphenous artery segments used for functional and Western blot studies were dissected from perfusion fixed rats (1% paraformaldehyde, 3% glutaraldehyde in 0.1 mM sodium cacodylate buffer, with 10 mM betaine, pH 7.4). Serial transverse sections (~100 nm thick) totaling ~5 μm of vessel length were cut, and MEGJs and their surrounding endothelial and smooth muscle cell (SMC) regions were counted and imaged at ~10,000 to 40,000 on a Phillips 7100 transmission electron microscope at 16-megapixel resolution (camera from Scientific Instruments and Applications, Duluth, GA). Quantitative wall properties were made from measurements of vessel cross-sections cut 90° perpendicular to the longitudinal vessel axis from ultrastructural montages taken at ×1500 to 2500 at 16-megapixel resolution. CellIR software (Olympus) was used for gross quantitative measurements. In Fig. 6E, wall properties of control and saphenous artery were n = 6, each from a different animal. MEGJ quantification is from n = 4, each from a different animal (Sandow et al., 2004). The number of SMC layers and medial cross-sectional area were determined from the mean of four points 90° apart for each vessel, the former as all ≥5-μm-long SMC profiles (Sandow et al., 2002, 2003, 2004).

**Statistical Analysis.** Agonist concentrations causing half-maximal responses (EC\(_{50}\) value) were calculated by using nonlinear regression analysis and expressed as the negative logarithm of the molar concentration (pEC\(_{50}\) values). Percentage vasodilation evoked by [agonist] was taken as the maximum (peak) responses. All data are expressed as mean ± S.E.M. from n experiments and were determined by one-way analysis of variance followed by Dunnett’s post test for multiple comparisons or paired t tests for groups of two. P < 0.05 was considered significant.

**Drugs.** Vasodilator blockers/antagonists N-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), indomethacin, apamin, and carbenoxolone were purchased from Sigma-Aldrich (Castle Hill, Australia). TRAM-34 was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Connexin-mimetic “Gap” peptides, \(\text{Gap}26\), \(\text{Gap}27\), and \(\text{Gap}27\), (Australian National University, Canberra, Australia). Scrambled peptide (CNVDTAGKAPTSA) was provided by Caryl Hill (WHO, Nairobi, Kenya). CyPPA was provided by Palle Christophersen (NeuroSearch, Ballerup, Denmark).

**Results**

**K\(_{Ca}\) Activity Is Present in Obese but Absent in Control Rat Saphenous Artery.** Phentylephrine-induced constriction and ACh-mediated dilation were unaltered in control compared with obese rat saphenous artery (Fig. 1A; Tables 2 and 3). In saphenous arteries from control and obese rats indomethacin (10 μM) alone had no effect on endothelium-dependent vasodilation to ACh compared with vehicle alone (pEC\(_{50}\) and E\(_{\text{max}}\) characteristics not being different from vehicle control; Table 3), and it was therefore not included in further protocols. However, the relative contribution of NO and K\(_{Ca}\) to endothelium-dependent dilation differed in obese and control arteries. Selective SK\(_{Ca}\), and IK\(_{Ca}\), block with apamin (50 nM) and TRAM-34 (1 μM), respectively, which, in
Altered Endothelial Function in Obesity

**Fig. 1.** KCa mediate endothelium-dependent vasodilation in obese rat, but not control saphenous artery. Saphenous arteries were mounted in a pressure myograph and preconstricted with phenylephrine (1 μM; 80% of maximum constriction), and subsequent responses to ACh were assessed. A, apamin (50 nM) and TRAM-34 (1 μM), in combination, significantly inhibit ACh-mediated dilation in obese rat artery (n = 5; *P < 0.05 indicates difference in pEC50 relative to vehicle control; Table 3), but had no effect in control (n = 6, each). B, ACh-mediated dilation in control (n = 5) was blocked with L-NAME (100 μM) and ODQ (10 μM), whereas in obese rat artery (n = 5), L-NAME and ODQ cause a significant rightward shift in the ACh concentration-response curve, although they did not fully block dilation (Table 3). C, remaining dilation in obese artery (n = 4) and full dilation in control (n = 6), was blocked by L-NAME and ODQ, in addition to apamin and TRAM-34. †, P < 0.05 indicates difference in Emax relative to vehicle control.

TABLE 2
pEC50 and Emax characteristics for phenylephrine-mediated constriction

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC50</td>
<td>Emax</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.8 ± 0.2</td>
<td>22.9 ± 7.4</td>
</tr>
</tbody>
</table>

In obese rat arteries, L-NAME and ODQ caused a 2-log unit rightward shift in the ACh concentration-response curve (P < 0.05; Fig. 1B; Table 3). However, in these vessels the sensitivity to L-NAME and ODQ was significantly less than in control (P < 0.05; Fig. 1B; Table 3). In both control and obese vessels, ACh-mediated dilation was blocked in the presence of L-NAME and ODQ plus apamin and TRAM-34 (P < 0.05; Fig. 1C).

**Altered IKCa Expression in Obese Rat Saphenous Artery.** To determine whether the change in endothelium-dependent dilation in the saphenous artery of diet-induced obese rats was accompanied by a change in SKCa and IKCa (represented by SK3 and IK1, respectively) expression, Western blots of membranes extracted from whole arteries were probed with characterized antibodies (see also Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005; Sandow et al., 2006) (Fig. 2; Supplemental Figs. S3–S6; Supplemental Table S2). SK3 protein expression was detected as a faint high molecular weight complex in control and obese rat arteries (Supplemental Figs. S3 and S5), whereas IK1 protein expression was ~3-fold higher in arteries from obese rats compared with control rats (P < 0.05; Figs. 2 and 3A; Supplemental Fig. S6; Supplemental Table S4).

IK1 appeared as a series of smeared bands (Fig. 2A), characteristic of glycosylated and/or hydrophobic membrane proteins possessing multiple phosphorylation sites, including a high molecular mass complex (>220 kDa), which represents the functional homotetrameric channel assembled in the membrane and was therefore used to quantify IK1 protein expression (Figs. 2 and 3A; Supplemental Results and Discussion; Supplemental Fig. S6; Supplemental Table S4). Some potassium channels, including SKCa and IKCa, when assembled as homotetrameric complexes, are resistant to dissociation by heat and detergents and migrate in SDS-polyacrylamide gel electrophoresis gels as a smeared complex. Thus, as has been demonstrated (Fig. 1C in Boettger et al., 2002; Fig. 3D in Chen et al., 2004; Fig. 1 in Mongan et al., 2005; Fig. 4C in Absi et al., 2007), IKCa appears as a series of smeared bands between ~50 and ~250 kDa, which was also the case in the present study (Fig. 2A). This is also reflected by the specific location and block of bands in SKCa- and IKCa-transfected cells using the same antibodies as the present study (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005), with said data mimicking that in the present study, being further confirmation of antibody specificity in the present study. More simply stated, antibody specificity in the present study is reflected by mimic of the specific location and block of bands in SKCa- and IKCa-transfected cells (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005). For further details see Supplemental Discussion, including additional references.

The distribution of SK3 and IK1 protein in whole arteries...
was examined by conventional confocal immunohistochemistry using characterized antibodies. SK3 was not detected with immunohistochemistry; although as a positive control, with the same protocol, it was present in proximal mesenteric artery endothelium (Supplemental Fig. S2, Inset). In both arteries from control and obese rats, IK1 was expressed in the endothelium, but not the smooth muscle, at a diffuse low level. However, IK1 distribution was altered in arteries from obese compared with control rats (Fig. 3, B ii, iii, v, and vi and C). Bright discrete IK1 densities (IK1 “plaques”) were present in control and obese rat arteries, although the size and distribution of these densities differed in control and obese, control having a larger number of small IK1 plaques compared with obese, where larger IK1 plaques were present (Fig. 3, B ii and iii compare v and vi and C). In addition, in arteries from obese animals, a greater number of IK1 plaques were associated with internal elastic lamina (IEL) holes compared with control (Fig. 3C; \( P < 0.05 \)). For antibody control details, see Fig. 3B, Insets and Supplemental Fig. S2.

**IKCa Mediate Endothelium-Dependent Vasodilation in Obese Rat Saphenous Artery.** The apparent lack of SK3 expression and the increase in IK1 in obese rat artery

<table>
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<tr>
<th></th>
<th>pEC(_{50})</th>
<th>( E_{\text{max}} )</th>
<th>n</th>
<th></th>
<th>pEC(_{50})</th>
<th>( E_{\text{max}} )</th>
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<td>Vehicle</td>
<td>7.1 ± 0.1</td>
<td>99.2 ± 0.6</td>
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<td>7.3 ± 0.03</td>
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<td>7.2 ± 0.2</td>
<td>96.6 ± 1.2</td>
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<tr>
<td>Apamin + TRAM-34</td>
<td>7.3 ± 0.1</td>
<td>97.6 ± 1.9</td>
<td>6</td>
<td></td>
<td>6.1 ± 0.1*†</td>
<td>96.7 ± 1.8</td>
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<tr>
<td>l-NAME + ODQ</td>
<td>n/a</td>
<td>22.1 ± 9.1*</td>
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<td>5.2 ± 0.1*</td>
<td>89.4 ± 1.1*†</td>
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<td>l-NAME + ODQ + Apamin + TRAM-34</td>
<td>6.7 ± 0.5</td>
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<td>6.4 ± 0.1*</td>
<td>94.2 ± 3.6</td>
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<tr>
<td>Vehicle</td>
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<td>6.0 ± 0.1*</td>
<td>95.5 ± 1.8</td>
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<tr>
<td>Gap peptides</td>
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<td>5.3 ± 0.3*</td>
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<td></td>
<td>6.9 ± 0.1</td>
<td>98.0 ± 1.0</td>
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<td>Carbenoxolone + TRAM-34</td>
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<td></td>
<td>5.1 ± 0.2*</td>
<td>78.9 ± 8.4*</td>
<td>4</td>
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\( n/a \), not applicable.

* \( P < 0.05 \) compared with vehicle treatment.

† \( P < 0.05 \) compared with control saphenous artery treatment.
suggest that KCa-mediated dilation in obese rat saphenous artery is mediated by IKCa alone. Thus, to examine the potential individual role of SKCa and IKCa, obese rat vessels were exposed to apamin and TRAM-34 separately. Apamin (50 and 100 nM) alone had no effect on ACh-mediated dilation (Fig. 4A; Table 3) with no difference in the effect of these two concentrations compared with vehicle alone, in artery from obese rat (Table 3; \( P < 0.05 \)). In contrast, TRAM-34 (1 \( \mu M \)) alone attenuated dilation (0.8 log units; \( P < 0.05 \); Table 3), compared with vehicle-exposed obese rat vessels (Fig. 4A; Table 3).

In support of the preceding apamin and TRAM-34 data, in both control and obese rat vessels, the concentration-selective IKCa activator, 1-EBIO (Kusama et al., 2005) evoked significant dilation that was not different between arteries from control and obese rats (\( P < 0.05 \); Fig. 4A; B and C). This response was subsequently blocked in the presence of TRAM-34 (1 \( \mu M \)) in artery from both control and obese rats (Fig. 4B). Concentration response data to 1-EBIO (1–300 \( \mu M \)) showed that a maximal IKCa-mediated response was elicited at 300 \( \mu M \) (Fig. 4C), as shown in previous studies (Kusama et al., 2005), in artery from both control and obese rats. It is noteworthy that whereas 1-EBIO elicited dilation in both control and obese rat vessels ACh produced IKCa-dependent dilation only in obese rats (Fig. 1), thus demonstrating that although IKCa can be activated by direct stimulation with 1-EBIO it does not contribute to ACh-mediated physiological function. In contrast to IKCa activation, the SKCa activator CyPPA (Hougaard et al., 2007; 0.01–30 \( \mu M \), and 1–30 \( \mu M \) for artery from control and obese rats, respectively) failed to dilate saphenous vessels (Fig. 4, B and D). It is noteworthy that in rat mesenteric artery experiments (Haddock et al., 2008) CyPPA (10 \( \mu M \)) elicited a 27 ± 13% dilation of control diameter (\( n = 3 \)), which was blocked by subsequent apamin (50 nM) application.

**Gap Junction Blockers Reduce Endothelium-Dependent Vasodilation in Obese Rat Saphenous Artery.** The putative gap junction blockers, carbenoxolone and Cx-mimetic “Gap” peptides, were used to examine MEGJ mediation of endothelium-dependent dilation. Exposure to carbenoxolone (100 \( \mu M \)) for 30 min had no effect on ACh-mediated dilation in control artery (Fig. 5A). In contrast, carbenoxolone significantly attenuated ACh-mediated dilation in obese rat artery (1 log unit; \( P < 0.05 \); Table 3), compared with vehicle-exposed vessels (Fig. 5B; Table 2). In a similar manner, 45-min incubation in the triple Gap peptide combination ([43]Gap26, [48]Gap27, and [37,42]Gap27; 100 \( \mu M \) each) also inhibited dilation to ACh (1.7 log units; \( E_{\text{max}} \) reduced 53%; \( P < 0.05 \); Table 3).

![Fig. 3. IKCa (IK1) expression and function is altered in obese rat saphenous artery.](image-url)

**C**

<table>
<thead>
<tr>
<th>IEL holes / IKCa plaques at IEL</th>
<th>IKCa plaques not at IEL</th>
<th>IKCa plaque size (( \mu m^2 ))</th>
</tr>
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<tbody>
<tr>
<td>10^3 ( \mu m^2 )</td>
<td>10^3 ( \mu m^2 )</td>
<td>size (( \mu m^2 ))</td>
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<tr>
<td>Control</td>
<td>9.0 ± 1.5</td>
<td>3.3 ± 0.8</td>
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<tr>
<td>(4)(^*) (at ~37% of IEL holes)</td>
<td>(~83% of IKCa plaques)</td>
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<td>Obese</td>
<td>6.4 ± 0.5(^*)</td>
<td>5.8 ± 0.4(^*)</td>
</tr>
<tr>
<td>(5)(^*) (at ~91% of IEL holes)</td>
<td>(~9% of IKCa plaques)</td>
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3). Incubation in control scrambled peptide (300 μM) was not significantly different from vehicle (Fig. 5B; Table 3). The maximum inhibitory effect of the Gap peptide combination was 35% greater than that of carbenoxolone (P < 0.05; Fig. 5B; Table 3).

Combined exposure to carbenoxolone (100 μM) and TRAM-34 (1 μM) had a cumulative inhibitory effect on ACh-mediated dilation in obese rat artery (Table 3), causing greater inhibition compared with carbenoxolone or TRAM-34 alone (Fig. 5C).

Myoendothelial gap junctions are frequent in obese rat saphenous artery, but rare in control artery. Serial section electron microscopy was used to examine MEGJ incidence (Fig. 6, A and B) and general vessel characteristics. In control rat arteries, MEGJs were rare, being closely associated with ~3% of IEL holes (P < 0.05; Fig. 6C–E). In arteries from obese rats, MEGJ incidence was ~12-fold higher than control (Fig. 6E), with ~55% of IEL holes having MEGJs. Of interest, the endothelial "head" of MEGJ-related projections in obese rat saphenous artery were multilobed, suggestive of a larger surface area (Fig. 6, A and B), which is consistent with larger IK plaque size (~7-fold) in obese compared with control rats (Fig. 3C). Furthermore, the number of SMC layers in obese rat vessels was significantly greater than control (P < 0.05; Fig. 6E), although medial cross-sectional area and media to lumen ratio did not differ (P > 0.05).

**Discussion**

The present study demonstrates that changes in the mechanism of endothelium-dependent vasodilation occur in the small saphenous artery branch of the diet-induced obese rat. As a result of a prolonged high-fat diet, obesity results in an up-regulation of IKCa and MEGJ-mediated activity, thereby maintaining vasodilator function. In contrast, under normal conditions in this vessel, such activity is entirely NO-mediated. Like the rat mesenteric artery (Mather et al., 2005; Dora et al., 2008), EDH-type vasodilator activity in the obese rat saphenous artery depends on specialized myoendothelial microdomain communication sites, where IKCa and MEGJs are localized in close proximity. Such activity is absent in the saphenous artery of normal lean littermate control rats.

In the obese rat saphenous artery, up-regulation of IKCa occurs in conjunction with recruitment and redistribution of the channel, as an increased association with IEL holes, which reflects the functional contribution of IKCa that is blocked by TRAM-34 and activated by 1-EBIO. Although IKCa and MEGJ-dependent activity is absent in control where it is not activated by ACh, dilation to 1-EBIO occurs in both control and obese rat vessels. This suggests that whereas IKCa in control does not contribute to the functional response evoked by ACh, it can be activated by direct stimulation with a concentration-selective agonist (Kusama et al., 2005). The latter observation probably reflects the lower expression and localization of IKCa at MEGJ-associated projections in control, the site of their primary microdomain-dependent activity.

In arteries from both control and obese rats, functional endothelial SKCa is absent. Apamin had no effect on ACh-mediated dilation, and direct activation of SKCa with CyPPA also had no effect, an observation further supported by the lack of functional SK3 detection with Western blotting and immunohistochemistry. The detection of SK3 protein as a monomer, but not as a high molecular weight complex on Western blots, suggests that SK3 is present in the saphenous artery, but not as a functional homotetrameric complex assembled in the membrane. Of interest, in a similar manner to obesity, a dominant role for IKCa, as opposed to SKCa in EDH has also recently been shown in mice by using a double knockout strategy (Brähler et al., 2009).

In control adult rat saphenous artery, an absence of MEGJs correlates with an absence of EDH (Sandow et al., 2002, 2004). As with IKCa, MEGJ up-regulation and incidence correlates with their functional contribution to EDH-type vasodilation in obese rat saphenous artery. Indeed, MEGJ coupling associated with EDH is present in juvenile
with exposure with either carbenoxolone or TRAM-34 alone (n > 10), resulted in significantly greater inhibition of ACh-mediated dilation in obese rat vessels compared to control (M) and TRAM-34 (1 μM) each in combination; response to scrambled peptide (300 μM) was the same as vehicle. The inhibitory effect of Gap peptides was greater than carbenoxolone mimics Cx43 block in astroglial cells (Benfenati et al., 2009), in a similar manner to its action in rat mesenteric artery (compare carbenoxolone data and interpretation in Tare et al., 2002; Dora et al., 2008). Thus, these nonspecific actions probably reflect some of their inhibitory effects in the present study. Nonetheless, at present these gap junction “blockers” are the most practical option available for examining gap junction effects. Of interest, in obese rat saphenous artery Gap peptides have a greater inhibitory effect on ACh-mediated dilation than carbenoxolone. The differential effect of these substances is consistent with either a more effective gap junction block or a greater nonspecific action of the peptides.

Of interest, in contrast to several vascular disease states (Luff et al., 2005), sympathetic innervation density is unchanged in obese rat saphenous artery (Supplemental Table S1), suggesting that sympathetic plexus density does not contribute to the mild hypertension in the diet-induced rodent model (Velkoska et al., 2005) examined in the present study.

In rat and mouse mesenteric artery, spatial and temporal modulation of calcium dynamics is critical for vascular function with sites of calcium release, targets of calcium action (such as IKCa), and gap junction Cxs being critical (Mather et al., 2005; Ledoux et al., 2008). In obese rat saphenous artery, the reliance of endothelium-dependent vasodilation on IKCa and MEGJs is consistent with the redistribution and accumulation of IKCa to IEL holes and the concomitant increase in MEGJ-associated projection density. The net result of the IKCa-MEGJ-dependent dilation is probably caused by either disparate or integrated IKCa and MEGJ Cx-related activity. Indeed, the cumulative effect of blocking IKCa and MEGJs on endothelium-dependent dilation of obese rat arteries suggests that, regardless of their close spatial association, these two mechanisms may function independently of one another, being dependent on transfer of current and localized release of Ca2+ and K+.

The IKCa-MEGJ mechanism, as a key component of EDH-mediated vasodilation, is absent in normal adult rat saphenous artery and up-regulated to compensate for diminished NO activity in arteries from obese rats. Although the present data support divergent MEGJ ( mechanism 1 in Supplemental Fig. S7) and IKCa (mechanism 2 in Supplemental Fig. S7)...

Fig. 5. Gap junctions contribute to endothelium-dependent vasodilation in obese rat saphenous artery. A, the putative gap junction uncoupler carbenoxolone (100 μM) had no effect on ACh-mediated dilation in control (n = 4). B, in contrast, carbenoxolone (100 μM) and Cx-mimetic Gap peptides (46,49Gap26, 46Gap27, and 37,43Gap27; 100 μM each in combination) significantly inhibit ACh-mediated dilation in obese rat artery (*, P < 0.05 indicates difference in pEC50 values relative to vehicle control; n = 4–5; Table 3). Response to scrambled peptide (300 μM) was the same as vehicle. The inhibitory effect of Gap peptides was greater than carbenoxolone (†, P < 0.05 indicates difference in Fmax relative to arteries exposed to carbenoxolone; Table 3). C, exposure to carbenoxolone (100 μM) and TRAM-34 (1 μM) had a cumulative effect, causing significantly greater inhibition of ACh-mediated dilation in obese rat vessels compared with exposure with either carbenoxolone or TRAM-34 alone (n = 4–12; ‡, P < 0.05 indicates difference in pEC50 values relative to either carbenoxolone or TRAM-34 treatment alone; Table 3).
contributions to EDH-type dilation, we hypothesize that the discrete microdomains at MEGJ-associated projections facilitate an integrated EDH-type response. Indeed, in theory such myoendothelial microdomain activity may function in three ways: a MEGJ-Cx-dependent signaling site, an IKCa-dependent signaling site, or a combination of the two, thus conferring considerable potential for functional heterogeneity. Such plasticity of myoendothelial coupling mechanisms represents a significant potential target for therapeutic intervention.

Acknowledgments

We thank Mark Chen (GliaXoSmithKline, Stevenage, United Kingdom) for SK3 and IK1 antibodies, Palle Christophersen (NeuroSEARCH, Ballerup, Denmark) for CyPAPA, and Caryl Hill (Australian National University, Canberra, Australia) for scrambled peptides.

References


Kusama N, Kajikuri J, Yamamoto T, Watanabe Y, Suzuki Y, Katsuya H, and Itob T

<table>
<thead>
<tr>
<th>No. SMC layers*</th>
<th>Medial CSA (µm²)</th>
<th>Media to lumen ratio (x10²)</th>
<th>IEL holes / 10⁴ µm²</th>
<th>MEGJs / 10⁴ µm²</th>
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<tr>
<td>Control</td>
<td>4.4 ± 0.1 (6)</td>
<td>7923 ± 476 (6)</td>
<td>4.85 ± 0.14 (6)</td>
<td>9.0 ± 1.5 (4)</td>
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<td>Obese</td>
<td>5.2 ± 0.1* (6)</td>
<td>8005 ± 273 (6)</td>
<td>4.70 ± 0.11 (6)</td>
<td>6.4 ± 0.5* (5)</td>
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*For definition of n, in parenthesis, see Online Supplementary Methods. **P<0.05, significant cf. control.**

Fig. 6. MEGJ and IEL hole characteristics in obese rat saphenous artery. A–D, serial section ultrastructural analysis shows MEGJs on endothelial cell (EC) projections (A and B and insets) and IEL holes (asterisk, C and D) without MEGJ-associated projections in control and obese rat arteries (example, C and D, from obese artery; see also Fig 3B). E, MEGJs are ~12-fold higher in density in obese compared with control. As found previously for some MEGJ-associated projections is multilobed (examples with asterisks, A and B); perhaps providing greater surface area for localized Ca²⁺ channel activity compared with nonlobed projections. Such sites have characteristic pentalaminar MEGJ morphology (arrows and insets) and apparent localized IKCa (IK1) densities (Fig 3B), as shown previously in rat caudal cerebral, mesenteric and juvenile saphenous artery (Sandow et al., 2006, 2009b). Bars, A and B, 1 µm; insets, 100 nm; C and D, 2 µm.


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